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RocR, a Novel Regulatory Protein Controlling Arginine Utilization in *Bacillus subtilis*, Belongs to the NtrC/NifA Family of Transcriptional Activators

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Bacillus subtilis **can use ammonium and various amino acids as sole nitrogen sources. The utilization of arginine or ornithine is abolished in a sigma L-deficient strain of *B. subtilis*, indicating that one or several genes involved in this pathway are transcribed by a sigma L-RNA polymerase holoenzyme. Three *B. subtilis* genes, called *rocA*, *rocB*, and *rocC*, which seem to form an operon, were found near the *sacTPA* locus (P. Glaser, F. Kunst, M. Arnaud, M.-P. Coudart, W. Gonzales, M.-F. Hullo, M. Ionescu, B. Lubochinsky, L. Marcelino, I. Moszer, E. Presecan, M. Santana, E. Schneider, J. Schweizer, A. Vertes, G. Rapoport, and A. Danchin, Mol. Microbiol. 10:371–384, 1993). The expression of this putative operon is induced by arginine and is sigma L dependent. Mutants impaired in the transcription of *rocA* were obtained. One of these mutants was used as recipient to clone and sequence a new regulatory gene, called *rocR*. This gene encodes a polypeptide of 52 kDa which belongs to the NtrC/NifA family of transcriptional activators. Upstream activating sequences highly similar to those of NtrC in *Escherichia coli* were also identified upstream from the *rocABC* genes. A *B. subtilis* strain containing a *rocR* null mutation is unable to use arginine as the sole nitrogen source, indicating that RocR is a positive regulator of arginine catabolism. After LevR, RocR is the second example of an activator stimulating sigma 54-dependent promoters in gram-positive bacteria.**

Bacillus subtilis can use ammonium or several amino acids as sole nitrogen sources (8, 11, 20). Among these, arginine can allow rapid growth in mineral medium. Bacteria use various strategies to degrade arginine, and multiple catabolic pathways frequently appear in the same bacterium, as observed in *Pseudomonas putida* (59). In *B. subtilis*, arginine is converted to glutamate by a series of three enzymatically catalyzed steps called the arginase pathway (8). Arginine is cleaved by arginase to give ornithine, the latter being converted to glutamate semialdehyde by ornithine aminotransferase. Finally, the conversion of glutamate semialdehyde to glutamate is catalyzed by a pyrroline-5-carboxylate dehydrogenase. This last reaction is shared with the proline catabolic pathway. The arginase metabolic pathway has been the subject of physiological and genetic studies. Synthesis of arginase and ornithine aminotransferase is induced in the presence of arginine, ornithine, or proline in the growth medium (9). Mutants resistant to arginine hydroxamate have been isolated and exhibit alterations in arginase and ornithine aminotransferase activities. It was concluded that these mutants could be affected in regulatory genes controlling arginine catabolism (7, 9, 25).

In *B. subtilis*, arginine represses the production of arginine biosynthetic enzymes and activates the arginine catabolism genes via a regulatory gene called *ahrC* (45). This gene encodes a product which is similar to the *Escherichia coli* ArgR repressor (48). The purified product of *ahrC* is a hexamer which binds in vitro to a DNA fragment encompassing the promoter of the *argCAEBD* biosynthetic genes (12). However, the mechanism by which AhrC activates arginine catabolism is not understood, and little is known at the molecular level concerning the regulation of synthesis of the enzymes of the arginase pathway.

We have recently shown that the utilization of arginine or ornithine as the sole nitrogen source is abolished in a sigma L-deficient strain of *B. subtilis*. This sigma factor has previously been shown to be an equivalent of sigma 54 present in gram-negative bacteria (14). This result indicates that one or several genes of the arginine degradative pathway are transcribed by a sigma L-RNA polymerase holoenzyme. Three candidate genes for this pathway, *ipa76d*, *ipa77d*, and *ipa78d*, which seem to form an operon, were found 25 kb downstream of the *sacTPA* locus (23). The expression of these genes, designated *rocA*, *rocB*, and *rocC*, is sigma L dependent and induced by arginine in the growth medium. The first gene, *rocA*, encodes a pyrroline-5-carboxylate dehydrogenase, the third enzymatic step of the arginase pathway. The second gene, *rocB*, is of unknown function, while the third gene product (RocC) is similar to members of a large family of amino acid transport proteins (57). To obtain further information concerning the regulation of the *rocABC* genes in *B. subtilis*, mutants impaired in the transcription of these genes were selected. Using one of these mutant strains, the corresponding wild-type gene was cloned by complementation experiments. This new regulatory gene, called *rocR*, controls arginine catabolism.

MATERIALS AND METHODS

Strains. The *B. subtilis* strains used in this study are listed in Table 1. *E. coli* K-12 strain TG1 [Δ(*lacZ-proAB*) *supE thi*

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TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype or description	Reference or source
168	*trpC2*	4
QB169	*trpC2 sacL8*	31
QB944	*trpC2 purA16 cysA14*	15
QB5176	*trpC2 levR::aphA3 amyE::*(pΔB*levD-lacZ*) *cat*	39
QB5505	*trpC2 sigL::aphA3*	14
QB5510	*trpC2 rocA-lacZ sacL8*	This study
QB5514	*trpC2 rocA-lacZ sacL8 rocR*	This study
QB5516	*trpC2 rocA-lacZ sacL8 sigL::aphA3*	This study
QB5519	*trpC2 rocA-lacZ sacL8 rocRΔ2::aphA3*[a]	This study
QB5533	*trpC2 rocRΔ3:::aphA3*[b]	This study
QB5534	*trpC2 rocA-lacZ sacL8 rocRΔ3::aphA3*[b]	This study
QB6049	*trpC2 sacXY::tet*	6
JH12586	*trpC2 abrB::cat*	50

[a] Corresponds to the *Hin*dIII deletion of *rocR* as shown in Fig. 5.
[b] Corresponds to the *Cla*I deletion of *rocR* as shown in Fig. 5. *aphA3* is the *S. faecalis* kanamycin resistance gene (60).

hsdD5/F' *traD36 proA*$^+$ *proB*$^+$ *lac*I^q *lacZ*ΔM15] was used for plasmid constructions and as a host for M13 bacteriophages. Standard procedures were used to transform *E. coli* (53), and selections were carried out on LB plates (53) supplemented with ampicillin plus chloramphenicol (50 and 2.5 μg/ml, respectively) or ampicillin plus kanamycin (100 and 5 μg/ml, respectively). *B. subtilis* transformation was performed as previously described, using plasmid or chromosomal DNA (4, 30), and selection was carried out on SP plates (3) containing chloramphenicol (5 μg/ml), kanamycin (5 μg/ml), or erythromycin (25 μg/ml).

Media. *E. coli* was grown in LB broth, and *B. subtilis* was grown in Penassay Antibiotic Medium 3 (Difco Laboratories, Detroit, Mich.) or MM minimal medium supplemented with 100 mg tryptophan per liter, 0.1% glucose, and 0.2% glutamate (MMGE) (4, 30).

Plasmids and plasmid construction. Plasmid pDIA5326 containing a *rocA-lacZ* transcriptional fusion was constructed as follows. A 1.8-kbp *Sal*I restriction fragment containing the promoter of the *rocA* gene was purified from the *Eco*RI-*Sal*I lambda phage (23) and cloned into the single *Sal*I site of pUC19. The cloned fragment was excised using the *Eco*RI and *Hin*dIII sites on each side of the polylinker and cloned into the corresponding sites of pDIA5307, leading to pDIA5326. pDIA5307 was constructed by inserting at the *Eco*RI and *Bam*HI sites of pJM783 the *Eco*RI-*Bgl*II polylinker excised from M13 TG131 (28, 49). In the resulting transcriptional fusion, the *lacZ* gene is fused downstream from codon 300 of the *rocA* gene and is expressed under the control of the *rocA* promoter.

PCR was used to introduce *Eco*RI restriction site at various positions upstream from *rocA*. PCR was performed by using plasmid pDIA5326 as the template, with one oligonucleotide (5'-GACGGGATCCTCGGTAAATGGTTCG-3') corresponding to the coding sequence of the *rocA* gene (codons 7 to 12) and the other one at various positions in the *rocA* promoter region. The *Eco*RI-*Bam*HI restriction fragments generated in this way were cloned between the *Eco*RI and *Bam*HI restriction sites of plasmid pAC7 (61), creating translational fusions between codon 12 of *rocA* and codon 8 of *lacZ*. Plasmids pSCA (ΔA), pSCB (ΔB), pSCC (ΔC), pSCD (ΔD), pSCE (ΔE), and pSCF (ΔF) were linearized by using the unique *Pst*I site, and the translational fusions were integrated into the chromosome by homologous recombination at the *amyE* locus of *B. subtilis* 168, using kanamycin selection (Table 1). Deleted fragments are shown in Fig. 3. Since DNA amplification by this method consistently led to misincorporation errors (0.1% under our conditions), the DNA sequences of the different PCR fragments were verified. Sequencing was carried out by cloning the *Eco*RI-*Bam*HI fragments into M13 vectors (42).

The *B. subtilis* gene bank used in this work was constructed as follows. Chromosomal DNA from *B. subtilis* 168 was partially restricted with *Sau*3A, and sized fragments (3 to 6 kb) were ligated at the dephosphorylated *Bam*HI site of plasmid pHT315 (5). *E. coli* TG1 was transformed with the ligation mixture, and plasmid DNA was extracted from a pool of 3,000 colonies.

DNA manipulations. Standard procedures were used to extract plasmids from *E. coli* (3, 53). Restriction enzymes, T4 DNA polymerase, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were used as recommended by the manufacturers. When necessary, 5' and 3' protruding ends were repaired to flush ends, using Klenow DNA polymerase, T4 DNA polymerase, and deoxyribonucleoside triphosphates. DNA fragments were purified from agarose gels by using a Prep-A-Gene kit (Bio-Rad Laboratories, Richmond, Calif.). DNA sequences were determined by using the dideoxy-chain termination method (54) and modified T7 DNA polymerase (U.S. Biochemical Corp., Cleveland, Ohio). Templates used for DNA sequencing were either single-stranded M13 phages, double-stranded plasmid minipreparations (29), or single-stranded PCR products produced through asymmetric amplification (27, 56). Chromosomal DNA was isolated from exponentially growing *B. subtilis* cells as previously described (46). Oligonucleotide primers were synthesized by the β-cyanoethylphosphoramidite method, using a Milligen/Biosearch Cyclone Plus synthesizer (Millipore, Inc., Burlington, Mass.), and used for amplification and sequencing reactions without purification.

PCRs (47, 52) were carried out by using *Thermus aquaticus* DNA polymerase as recommended by New England Biolabs, Inc. (Beverly, Mass.). Oligonucleotide primers used for PCR included mismatches allowing creation of *Eco*RI or *Bam*HI restriction sites. After an initial denaturation step of 10 min at 95°C, amplification was done for 25 rounds. The DNA was denatured at 95°C for 1 min, annealed at 55°C for 1 min, and extended at 72°C for 2 min. Samples were successively extracted with phenol and chloroform, ethanol precipitated, and digested with appropriate restriction enzymes before gel purification.

Reverse transcriptase mapping of the mRNA start point of the *rocA* gene. Total RNA was isolated from *B. subtilis* 168 grown in MMGE medium supplemented with tryptophan and with or without 20 mM arginine as the inducer. Exponentially growing cells were harvested at an optical density at 600 nm of 1, and the RNA was extracted as previously described (24). Primer extensions were carried out by using two different probes. A first ^{32}P-labelled DNA primer was synthesized from a recombinant M13 bacteriophage containing a *Hin*dIII-*Eco*RI restriction fragment purified from pDIA5326 and integrated into the corresponding sites of M13 mp18 double-stranded DNA.

Single-stranded DNA (0.5 μg) was hybridized with an 18-mer primer (5'-GATTTTGCGGTCCGTTTC-3') complementary to the *rocA* coding sequence (codons 47 to 52). The hybridization mixture was incubated for 20 min with 0.37 MBq of [α-^{32}P]dATP (15 TBq/mmol) in the presence of cold dGTP, dCTP, and dTTP and 5 U of the Klenow fragment of DNA polymerase. The DNA was then digested with *Hin*fI and heated at 95°C in the presence of formamide. A radioactive single-stranded DNA primer containing 83 bases complemen-

tary to codons 25 to 52 of the *rocA* gene was then purified by polyacrylamide gel electrophoresis (7%). In a second experiment, an oligonucleotide (5′-GGCCAATGACTCCCCAAAT GCAG-3′) complementary to the *rocA* coding sequence (codons 20 to 27) was labelled with 10 U of polynucleotide kinase and 0.37 MBq of [γ-^{32}P]ATP (15 TBq/mmol). Elongation of the DNA primers and analysis of the products were performed as previously described (38).

β-Galactosidase assays. *B. subtilis* cells containing *lacZ* fusions were grown in the indicated media. β-Galactosidase specific activities were determined as previously described and expressed as Miller units per milligram of protein (43, 46). The values indicated represent averages from at least three independent assays.

B. subtilis colonies expressing *lacZ* fusions were detected by overlaying colonies with 8 ml of soft agar (7.5 mg/ml) containing lysozyme (2 mg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (100 μg/ml).

Computations. Computations were performed at the National Center for Biotechnology Information by using the BLAST network service (2).

Nucleotide sequence accession number. The nucleotide sequence of the *rocR* gene reported here has been submitted to GenBank and assigned accession number L22006.

RESULTS

Control regions upstream from the *rocA* promoter. The *rocABC* genes were found near the *sacTPA* locus during the *B. subtilis* genome sequencing project (*roc* stands for arginine, ornithine catabolism) (23). Transcription of the *rocA* gene is induced by arginine in the growth medium. The transcription start site was mapped by primer extension using reverse transcriptase (Fig. 1 and 3). The promoter region was shown to contain a region consistent with the −12, −24 consensus sequence of sigma ^{54}-dependent promoters. Thirteen of eighteen residues are identical in the levanase operon and *rocA* promoters (Fig. 2). Indeed, the expression of the *rocA* gene depends on the *sigL* gene product, as shown below.

In gram-negative bacteria and for the *B. subtilis* levanase operon, sigma 54-dependent promoters are positively regulated and contain regulatory sequences, called upstream activating sequences (UASs), which are recognized by specific activators. Transcriptional activators usually bind to specific DNA sequences located far upstream of the transcriptional start site. The binding sequences are often inverted repeats that can be moved more than 1 kb away without losing their ability to allow transcriptional activation (10, 37, 41). To identify any such sequences associated with the *rocA* promoter, we created deletions ending upstream from the transcriptional start site. A set of DNA fragments from which part of the upstream region was missing was obtained by PCR synthesis (see Materials and Methods). These fragments were then cloned upstream from the *lacZ* gene (see Materials and Methods). Deletion end points are indicated in Fig. 3. Fusions were reintroduced as single copies at the *amyE* locus of *B. subtilis* 168. The levels of *lacZ* expression were assayed in these strains and are shown in Fig. 3. In ΔD, ΔE, or ΔF strains, *lacZ* expression was extremely low. In a ΔC strain, an intermediate level of *lacZ* expression was observed. In the absence of arginine in the growth medium, the β-galactosidase activities in all of the deletion strains were less than 5 U/mg of protein. This experiment indicates that DNA sequences around −165 with respect to the transcriptional start site are necessary for full induction of the putative *rocABC* operon. The sequence in this region is very similar to the sequence of the NtrC binding

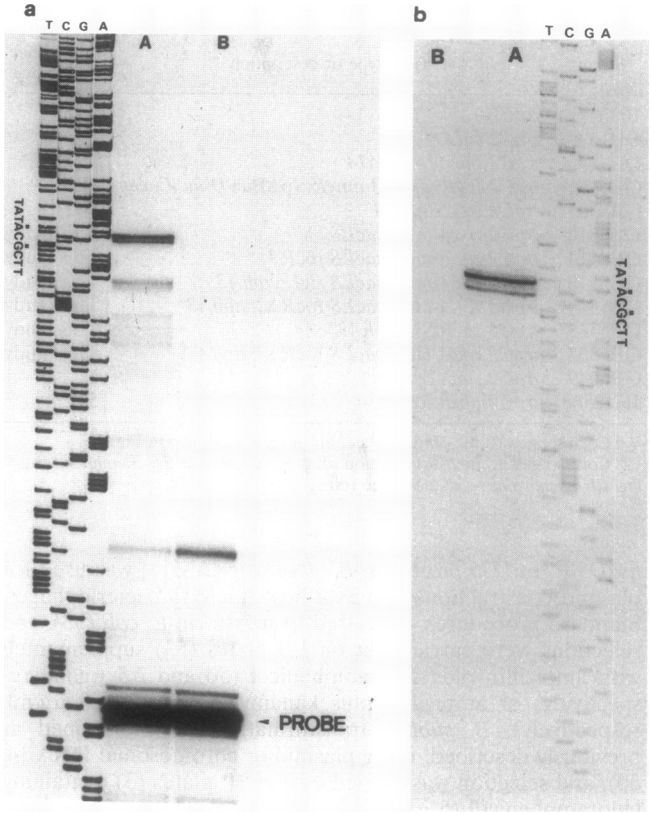


FIG. 1. Reverse transcriptase mapping of the transcriptional start site of the *rocA* gene. RNA was extracted from *B. subtilis* 168 grown in the presence (lanes A) or absence (lanes B) of 20 mM arginine. Positions of the cDNA-extended fragments were compared with those obtained by sequencing a M13 recombinant phage containing the promoter region with the oligonucleotide corresponding to the probe. (a) Primer extension experiment carried out with the M13 probe; (b) experiment done with the 23-mer oligonucleotide. In both cases, the same transcriptional start sites were obtained. The presence of additional bands visible in panel a probably corresponds to nonspecific hybridization, since they are also present under conditions in which *rocA* is not expressed (absence of arginine; see Table 2).

site in gram-negative bacteria, TGCACC(A/T)$_4$TGGTGCA (19). A second copy of this 17-base sequence is present between positions −112 and −132. Only 4 bases of 21 differ between these two sequences (Fig. 4). It seems likely that a positive regulator interacts with these upstream sequences, since deletion analysis showed a sharp decrease in the rate of β-galactosidase synthesis. This finding suggests that *B. subtilis* may contain an activator of the NtrC family which stimulates transcription of the *rocA* gene. We devised a strategy for cloning this regulatory gene, by isolating mutants defective in

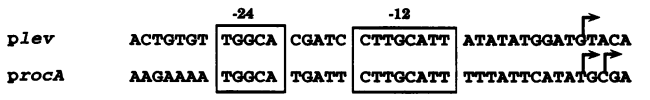
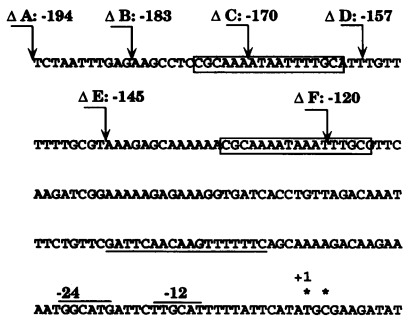


FIG. 2. Nucleotide sequences of promoter regions of the levanase operon (*plev*) and the *rocA* gene (*procA*). The transcriptional start sites are indicated by arrows. The *plev* start site was mapped by primer extension as previously described (38). Boxes indicate strictly conserved DNA sequences around positions −12 and −24 with respect to the transcriptional start sites.



Deletion Endpoint:	β-Galactosidase specific activity (Units/mg protein)
ΔA: -194	410
ΔB: -183	400
ΔC: -170	130
ΔD: -157	35
ΔE: -145	10
ΔF: -120	5

FIG. 3. Nucleotide sequence of the *rocA* upstream region. Deletion end points are indicated by vertical arrows and numbered with respect to the transcriptional start site (indicated by asterisks). −12, −24 sequences are indicated (upper line). A DNA sequence similar to the IHF consensus binding site is underlined. Boxed regions indicate putative UASs. The effects of upstream deletions on expression of the *rocA-lacZ* translational fusion are indicated. β-Galactosidase specific activities were determined in extracts prepared from exponentially growing cells in MMGE medium containing 20 mM arginine as the inducer.

the expression of the *rocA* gene. This procedure is described below.

Isolation of mutants impaired in the expression of the *rocA* gene in *B. subtilis*. *B. subtilis* QB5510 contains a *rocA-lacZ* transcriptional fusion. This strain was constructed as follows. Plasmid pDIA5326, which contains the transcriptional *rocA-lacZ* fusion (see Materials and Methods), cannot replicate in *B. subtilis*. It was introduced by transformation into *B. subtilis* QB169 (*sacL8*) to produce strain QB5510. Stable transformants were obtained following a single crossover event, leading to integration of the plasmid at the *rocABC* locus. This strain has a dark blue phenotype when plated on an MMGE–X-Gal plate containing 20 mM arginine as the inducer. β-Galactosidase activity was assayed in culture of this strain grown with or without inducer (Table 2). A *sigL* null mutation was introduced into strain 168, which contains the same *rocA-lacZ* fusion, to produce strain QB5516, and β-galactosidase activities were assayed (Table 2). Expression of the *rocA-lacZ* fusion was strongly dependent on the *sigL* gene product.

Four independent aliquots of strain QB5510 were mutagenized and plated on MMGE–X-Gal plates containing arginine. Twenty-seven colonies were white; of these, five mutants were probably affected in the *sigL* gene, since both the levanase and the *rocA* genes were not expressed. The presence of the constitutive allele of *levR* (*sacL8*) allowed convenient identification of mutants affected in levanase expression. Indeed, the constitutive expression of the levanase operon was much higher in the *sacL8* mutant than in the induced wild-type strain. Fourteen of the remaining mutations mapped very close to the *cat* gene of the integrated plasmid. Moreover, in these 14 mutants, levanase operon expression was not affected, as revealed by assaying sucrose hydrolysis (38). In five other mutants, *rocA* gene expression was still inducible by ornithine but not by arginine. This finding suggests that the conversion of arginine to the internal inducer was abolished in these mutants. It has previously been suggested that either pyrroline-5-carboxylate or glutamate semialdehyde, formed at an intermediate step at which the arginine and proline catabolic pathways converge, is the true inducer. The step impaired in these mutants is probably the conversion of arginine to ornithine by the arginase (16, 33). The remaining three mutants, which were white on ornithine and on arginine–X-Gal plates, were studied.

β-Galactosidase was assayed in a culture of one of these mutants, QB5514 (Table 2). This mutant did not express *lacZ*. The gene affected in QB5514 was named *rocR*. The wild-type *rocR* gene was cloned by complementation as follows.

Cloning and characterization of the *rocR* gene. Strain QB5514 was transformed with a library of *B. subtilis* DNA established in *E. coli*, using the shuttle plasmid pHT315 (35). Transformants were selected on MMGE–erythromycin–X-Gal plates containing 20 mM arginine. Five blue colonies were isolated from approximately 50,000 colonies. Plasmid DNA was extracted from cultures of each of these clones and reintroduced independently by transformation into QB5514. Transformants were isolated on SP-erythromycin plates and reisolated on MMGE–X-Gal plates containing 20 mM arginine. For each transformation (giving about 200 transformants), about 80% had a dark blue phenotype indicating a complementation leading to *lacZ* expression. The remaining white transformants were probably due to recombination between the cloned gene and the defective allele on the chromosome. These plasmids were introduced by transformation into *E. coli* for large-scale purification of DNA. The restriction map of one of these plasmids, pROC1, is shown in Fig. 5. To locate precisely the gene isolated in plasmid pROC1, a kanamycin cassette (*aphA3* gene from *Streptococcus faecalis*) (60) was

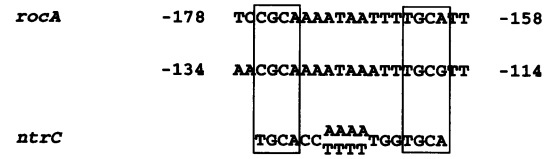


FIG. 4. Comparison of sequences upstream from the *rocA* gene with the consensus NtrC binding site. Highly conserved regions are boxed, and numbers indicate positions with respect to the transcriptional start sites.

TABLE 2. Effects of *rocR* and *sigL* mutations on *rocA-lacZ* expression

Strain	Relevant genotype	β-Galactosidase activity (Miller units/mg of protein)	
		− Arginine	+ Arginine
QB5510	*rocA-lacZ*	50	16,200
QB5514	*rocA-lacZ rocR*	10	15
QB5516	*rocA-lacZ sigL::aphA3*	50	55
QB5519	*rocA-lacZ rocR::aphA3* Δ2(*Hin*dIII)	10	180
QB5534	*rocA-lacZ rocR::aphA3* Δ3(*Cla*I)	15	10

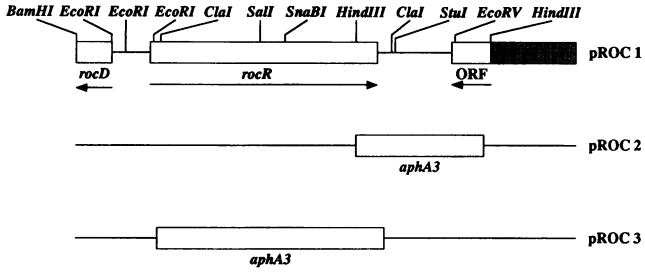


FIG. 5. Simplified restriction map of plasmids containing the cloned DNA fragments used in this work. pROC1, pROC2, and pROC3 are pHT315 derivatives. The *rocR* gene in pROC2 and pROC3 is partially deleted and interrupted by a DNA cassette containing the *aphA3* gene. The hatched box indicates a DNA fragment not sequenced. ORF, open reading frame.

introduced between the two *Hin*dIII restriction sites, leading to pROC2. Similarly, the kanamycin cassette was introduced between the two *Cla*I sites of pROC1, leading to pROC3. These kanamycin cassettes were reintroduced by a double crossover into the chromosome of QB5510, leading to strains QB5519 and QB5534, respectively. The expression of the *lacZ* gene was assayed in cultures of these strains (Table 2). *lacZ* gene expression remained weakly inducible in QB5519, but the induced level was reduced 100-fold compared with that obtained with the wild-type fusion strain (see Discussion). Deletion of the *Cla*I fragment in QB5534 strain abolished the expression of the *lacZ* gene. Thus, the *Cla*I and *Hin*dIII restriction sites map within the *rocR* gene. These experiments also indicate that the *rocR* gene product is necessary for a full induction of the *rocA* gene. We made the assumption that pROC1 plasmid contains a positive regulatory gene stimulating transcription of the *rocA* gene. To test this hypothesis, we examined the expression of the levanase operon, which is transcribed by a sigma L-dependent RNA polymerase (14) in the presence of multiple copies of pROC1. Indeed, if pROC1 encodes a regulatory protein with a central domain which interacts with sigma L-RNA polymerase holoenzyme to stimulate the *rocA* promoter, overexpression of RocR could also stimulate the levanase promoter through cross-activation. This type of phenomenon has previously been observed in other systems, including the *dctA* gene of *Rhizobium meliloti* (34). Strain QB5176 is deleted for the specific activator *levR* and contains a *levD-lacZ* fusion. This strain was transformed with pROC1. Expression of the *levD-lacZ* was assayed in the absence and presence of arginine (20 mM) as the inducer, giving values of 5 and 35 Miller units/mg of protein, respectively. This induced level is much lower than that obtained for the same *levD-lacZ* fusion in the presence of the cognate LevR regulator (790 Miller units/mg of protein) (41). Nevertheless, the sevenfold stimulation suggests that pROC1 contains a regulatory gene which, when present in multiple copies, stimulates transcription of the *rocA* gene and of the levanase operon.

The DNA sequence of the inserted fragment in pROC1 was established (see Materials and Methods). The sequence contains an open reading frame beginning at position 439 and ending with a TAA stop codon at position 1831. A DNA sequence 5′-AGGGAGGT-3′, presenting a good match with the consensus Shine-Dalgarno sequence, is followed by a GTG initiation codon. The open reading frame extends 18 nucleotides upstream from the proposed GTG start codon. Since no other start codon preceded by a ribosome binding site at a

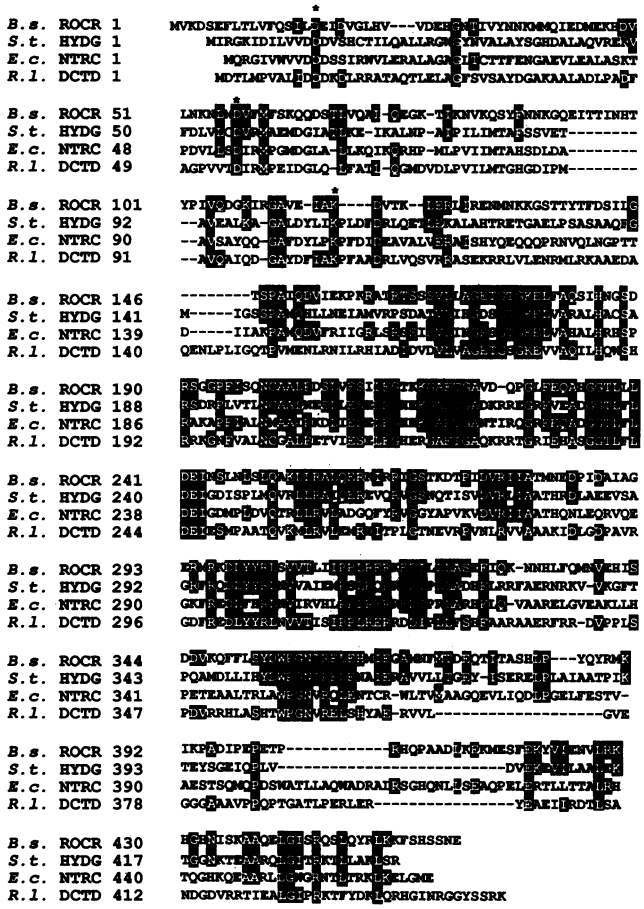


FIG. 6. Comparison of the amino acid sequence deduced from the nucleotide sequence of *rocR* with sequences of similar regulatory proteins. Identical residues are boxed, and numbers represent the positions of the residues in the respective proteins. Residues indicated by asterisks in the amino-terminal region are highly conserved among response regulators of two-component systems. The percentage identities between RocR from *B. subtilis* (B.s.) and HydG from *S. typhimurium* (S.t.) (58), NtrC from *E. coli* (E.c.) (44), and DctD from *R. leguminarium* (R.l.) (51) are 31, 28, and 27, respectively.

correct distance was present upstream, this GTG is presumably the translational initiation codon of the RocR polypeptide. The deduced polypeptide contains 461 residues with a calculated molecular weight of 52,152. A computer search revealed that this protein is similar to members of a family of activators of −12, −24 promoters. Figure 6 presents an alignment of the RocR polypeptide with HydG from *Salmonella typhimurium* (31% identity) (58), NtrC from *E. coli* (28% identity) (44), and DctD from *Rhizobium leguminosarum* (27% identity) (51).

This family of activators contains a region of 220 to 240 residues called the central domain. This domain is specifically required for the formation of open complexes between RNA polymerase and the −12, −24 promoter, probably by interacting with RNA polymerase or with sigma 54. The central domain also contains an ATP binding site which is present and well conserved in the RocR polypeptide (centered at position 175). RocR shows similarity with LevR of *B. subtilis* only in the central domain (34% identity over 200 residues) (13). All known NtrC/NifA type of activators also contain a helix-turn-helix (HTH) motif in the C-terminal part of the protein, and this motif is involved in the interaction with the UAS. The

TABLE 3. Doubling times of *rocR* and *sigL* mutants in minimal medium containing amonium or arginine nitrogen source

Strain	Doubling time (min) with indicated nitrogen source	
	NH_4Cl (20 mM)	Arginine (20 mM)
168	65	45
QB5505 Δ*sigL*	60	480
QB5533 Δ*rocR*	60	420

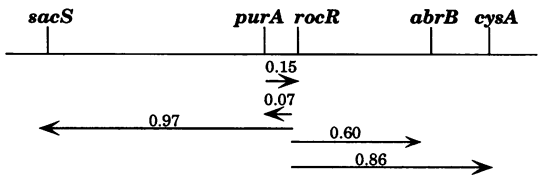


FIG. 7. Genetic mapping of the *rocR* locus of *B. subtilis*. The map was obtained from three-factor crosses by PBS1 transduction. Values represent 1 − cotransduction frequency. Arrows point from the selected to the nonselected markers.

Dodd and Egan method (17) was used for the prediction of potential HTH motifs in RocR. A standard deviation score of 5.5 was obtained for a region located between positions 431 and 453 of the RocR polypeptide, indicating that this region has a very high probability of being an HTH motif. HydG, NtrC, and DctD belong to the response regulator family of the two-component systems and share a conserved amino terminal domain of approximately 120 amino acids. This conserved domain contains at least three invariant amino acid residues, indicated with asterisks in Fig. 6. However, the overall similarity with RocR in this region is low (Fig. 6).

Upstream from the *rocR* gene, an open reading frame of 306 bp which is transcribed in opposite direction was found. The beginning of this open reading frame contains a putative ATG initiation codon preceded by a Shine-Dalgarno sequence 5′AAGGGGGAATT-3′. The deduced polypeptide contains 70 residues which are similar to the amino-terminal sequence of the ornithine aminotransferase from the yeast *Saccharomyces cerevisiae* (55% identity) and from humans (53% identity). A −12, −24 promoter is present 60 bp upstream from the Shine-Dalgarno sequence (not shown). This gene, which most probably encodes the ornithine aminotransferase enzyme of *B. subtilis*, was called *rocD* (see Discussion).

The end of an open reading frame was also found between the *Hin*dIII site and the *Eco*RV site downstream from *rocR* (Fig. 5). The deduced polypeptide of 81 residues is similar to the carboxy-terminal part of the heat shock protein HtrA from *S. typhimurium* (34% identity) (36). The function of this putative gene product is unknown.

Phenotype of *rocR* mutant in *B. subtilis*. *B. subtilis* QB5533 containing an *aphA3* gene inserted into the *rocR* gene was tested for growth in glucose minimal medium containing arginine as the sole nitrogen source (Table 3). The strain grew slowly: the doubling time was sevenfold longer than that observed in the presence of NH_4^+. Therefore, the *rocR* gene is involved in the control of one or more genes required for arginine catabolism (genes encoding arginase, ornithine aminotransferase, or permease). A strain in which the *sigL* gene is inactivated cannot grow in minimal medium containing arginine or ornithine, isoleucine, or valine as a nitrogen source (14). The *rocR* mutant was therefore tested for growth in presence of isoleucine or valine as the sole nitrogen source. The *rocR* null mutation did not affect the utilization of either amino acid as the sole nitrogen source (not shown).

Location of the *rocR* gene on the *B. subtilis* genetic map. The *rocR* gene was located on the genetic map by transduction crosses using bacteriophage PBS1. A PBS1 lysate was prepared from a culture of QB5533 containing an *aphA3* cassette inserted into the *rocR* gene. The PBS1 lysate was used to transduce *B. subtilis* QB944 (*cys14 purA16*). After selection for kanamycin resistance, linkage could be shown between *cysA* and *rocR* and between *purA* and *rocR*. The transducing lysate was also used to transduce QB6049 *sacXY*::*tet*, and linkage was shown between *rocR* and *sacXY*. To confirm the linkage observed with *cysA*, a recipient strain, JH12586 containing a *cat* gene inserted in the *abrB* gene, was transduced. Kanamycin-resistant clones were selected, and again linkage was observed between *rocR* and *abrB* (Fig. 7). These data map the *rocR* gene between *purA* and *cysA* close to the *purA* gene on the genetic map.

DISCUSSION

Multiple pathways for L-arginine utilization have been described in microorganisms, and the enzymes of the arginase pathway were found in *B. subtilis* several years ago (1, 7, 11, 33). However, very little was known about the regulation of biosynthesis of the enzymes of the arginase pathway in *B. subtilis*.

The *rocABC* genes were characterized during the *B. subtilis* genome sequencing project. The −12, −24 promoter of this putative operon is the second example of this type of promoter described in gram-positive bacteria. Activity measurements have shown that the *rocA* gene product is a pyrroline-5-carboxylate dehydrogenase (23). A *B. subtilis* strain containing a *rocA* null mutation still exhibits a residual pyrroline-5-carboxylate dehydrogenase activity representing 20% of the level observed in the induced wild-type strain. Furthermore, the *rocA* strain grows in minimal medium containing arginine as the sole nitrogen source (22). There have been similar findings in *Bacillus licheniformis*, in which two enzymes which catalyze the oxidation of pyrroline-5-carboxylate to glutamic acid have been characterized (16). The polypeptide encoded by the *rocC* gene displays similarities with a family of amino acid permeases. The function of the *rocB* gene product is unknown: it does not show any similarity to proteins in data banks (23).

We characterized a new regulatory gene, called *rocR*, which controls transcription of the *rocA* gene. This regulatory gene encodes a polypeptide of 52 kDa which belongs to the NtrC/NifA family of transcriptional activators. RocR and NtrC share a strong amino acid similarity, particularly in the central domain and in the carboxy-terminal region. The carboxy-terminal region of RocR is predicted to contain an HTH motif between residues 431 and 453. Residual induction of the *rocA-lacZ* fusion was observed in strain QB5519 containing a *Hin*dIII deletion which removes the last 46 codons of *rocR*, including the HTH motif. The activity of the truncated protein (with an intact central domain) is consistent with other previous reports of active truncated activators (13, 26).

In gram-negative bacteria, the regulation of nitrogen metabolism is controlled mainly at the transcriptional level by the nitrogen utilization system (37). The arginine, histidine, and glutamine permeases are positively controlled by NtrB and NtrC and transcribed by a sigma 54-associated RNA polymerase (19, 55). No functional equivalents of NtrB and NtrC have been found in gram-positive bacteria. NtrC and RocR

share strong sequences similarities; however, RocR appears to be specific for arginine catabolism, since isoleucine and valine utilization is not affected in a *rocR* null mutant.

In gram-negative bacteria, NtrC is regulated by NtrB, a protein kinase which is first autophosphorylated at a conserved histidine residue in an ATP-dependent reaction. This histidine residue is found in all members of sensor kinase family. In a second step, the phosphoryl group is transferred to an aspartate residue in the amino-terminal domain of the response regulator. Proteins of the response regulator family share a conserved amino-terminal domain of approximately 120 amino acids containing Asp residues. Corresponding Asp residues are also present in the RocR polypeptide.

The functions of the activators of −12, −24 promoters are controlled by different mechanisms. LevR in *B. subtilis* is probably inactivated by phosphorylation via the phosphotransferase system (40), NifA in *Klebsiella pneumoniae* is inactivated by high oxygen tension via the NifL polypeptide, and XylR of *P. putida* probably interacts directly with xylene (32). Whether RocR of *B. subtilis* is regulated by phosphorylation via an histidine kinase is yet unknown. In addition, arginine maybe required for the *rocR* transcription.

We characterized a DNA sequence 166 bp upstream from the *rocA* promoter and showed that it is involved in its transcription. This sequence is present in two copies and shares similarities to the NtrC binding site found in gram-negative species. The *glnAP2* promoter of *E. coli* is one of the best studied −12, −24 promoters. It contains two adjacent high-affinity NtrC binding sites centered 110 and 140 bp upstream from the transcriptional start site (18). Similar repeated DNA sequences are also present 78 and 146 bp upstream from the putative −12, −24 promoter of the *rocD* gene. Preliminary results have shown that expression of the *rocD* gene is induced by arginine, ornithine, or proline in the growth medium. Moreover, *rocD* transcription is sigma L and RocR dependent (21), and it is tempting to speculate that the sequences upstream from *rocABC* and *rocD* could be the target of RocR. Arginine utilization is abolished in a *rocR* mutant. Therefore, the RocR polypeptide seems to be a regulator of arginine catabolism. In gram-negative species, maximal activation of transcription depends on an additional protein called the integration host factor (IHF). This factor binds to a site located between the UAS and the promoter. It seems that IHF stimulates transcription by bending the DNA by more than 140°, allowing a direct contact between the sigma 54-RNA polymerase complex and the activator bound to the UAS. A DNA sequence similar to the consensus binding site of IHF has previously been found upstream from the levanase operon promoter (41). An IHF consensus site is also present upstream from the *rocA* promoter (Fig. 3). However, no IHF equivalent has yet been identified in *B. subtilis.*

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